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[DOCUMENT NAME] CLAIMS

[Claim 1]

A method of producing a DNA array, characterized by comprising the steps of:

- (1) preparing a mixture of DNA fragments in which cytosine to be methylated or methylated cytosine is exposed,
- (2) bringing the mixture of DNA fragments obtained in step (1) into contact with an antibody specific to cytosine or an antibody specific to methylated cytosine, and separating the mixture into a group consisting of DNA fragments which form an immunocomplex with the antibody and another group consisting of DNA fragments which do not react with the antibody,
- (3) identifying all or part of DNA fragments contained in each of the DNA fragment groups, and
- (4) arranging one or more nucleic acids capable of hybridizing with any one of the identified DNA fragments on a substrate.

[Claim 2]

The method according to claim 1, wherein the contact of the DNA fragment mixture with the antibody is carried out under conditions in which a monovalent binding is dissociated and a divalent binding is maintained in the antigen-antibody reaction.

[Claim 3]

The method according to claim 1 or 2, wherein the mixture of DNA fragments in which cytosine to be methylated or methylated cytosine is exposed is prepared by digesting genomic DNA with a restriction enzyme which can digest a DNA regardless of the presence or absence of methylation in a recognition site to generate a cohesive end containing cytosine to be methylated or methylated cytosine.

[Claim 4]

The method according to claim 1 or 2, wherein the mixture of single-stranded DNA fragments or partially single-stranded DNA fragments in which cytosine to be methylated or methylated cytosine is exposed is prepared by fragmenting genomic DNA and rendering the fragmented genomic DNAs fully or partially single-stranded.

[Claim 5]

The method according to claim 1 or 2, wherein a mixture of free DNA fragments contained in a biological sample or a

sample derived therefrom is used as the mixture of DNA fragments.

[Claim 6]

A DNA array obtainable by the method according to any one of claims 1 to 5.

[Claim 7]

A group of DNA fragments, characterized by comprising only any one of

- (1) a DNA fragment having cohesive ends containing cytosine to be methylated or methylated cytosine at both ends, wherein methylated cytosine is contained in both of the cohesive ends,
- (2) a DNA fragment having cohesive ends containing cytosine to be methylated or methylated cytosine at both ends, wherein methylated cytosine is contained in only one of the cohesive ends, or
- (3) a DNA fragment having cohesive ends containing cytosine to be methylated or methylated cytosine at both ends, wherein no methylated cytosine is contained in both of the cohesive ends.

[Claim 8]

A DNA array characterized in that one or more nucleic acids capable of hybridizing with all or part of DNA fragments contained in the group of DNA fragments of claim 7 are arranged on a substrate.

[Claim 9]

A method of analyzing DNA methylation in a DNA to be assayed, characterized by comprising the steps of:

- (1) preparing a mixture of DNA fragments in which cytosine to be methylated or methylated cytosine is exposed, from the DNA to be assayed,
- (2) bringing the mixture of DNA fragments obtained in the step (1) into contact with an antibody specific to cytosine or an antibody specific to methylated cytosine, and separating the mixture into a group consisting of DNA fragments which form an immunocomplex with the antibody and another group consisting of DNA fragments which do not react with the antibody, and
- (3) analyzing DNA fragments contained in the DNA-fragment group which forms an immunocomplex with the antibody or the DNA-fragment group which does not react with the antibody, using a DNA array.

A method of purifying a double-stranded DNA fragment having a cohesive end, characterized by bringing the double-stranded DNA fragment into contact with an antibody specific to a base contained in the cohesive end.

[DOCUMENT NAME] Specification

[TITLE OF THE INVENTIONS] DNA ARRAY FOR ANALYZING DNA METHYLATION, METHOD OF PRODUCING THE SAME AND METHOD OF ANALYZING DNA METHYLATION

[TECHNICAL FIELD]

[0001]

The present invention relates to a DNA array for analyzing DNA methylation, a method of producing the DNA array, and a method of analyzing DNA methylation. Further, the present invention relates to a method of purifying or obtaining a DNA fragment.

[BACKGROUND ART]

[0002]

Genetic information of organisms is integrated into DNAs. Although it is general knowledge that nucleotide sequences of these DNAs define all features of organisms, aging of genomic DNA is carried out in higher animals. Actually, DNA methylation of cytosine bases in DNAs is changed together with aging, and many diseases caused by the change are known. When genomic DNA is transplanted into an egg, the genomic DNA is demethylated and rejuvenated. This phenomenon has been confirmed by experiments using clone animals, and the regeneration of telomere length has been also confirmed in mice. The aging of the genome results in, for example, a suppression of a gene to be expressed or an expression of a gene to be inhibited. In many cancer cells, methylation in promoter regions which regulate gene expression is enhanced, and as a result, many tumor suppressor genes are inactivated.

[0003]

As above, methylation in DNA chains is an important index for various diseases including cancers. Further, since methylation is involved in the regulation of gene expression, methylation may be an index for evaluating, for example, an extent of cell differentiation. Under these circumstances, various methods for measuring methylation have been examined.

From another viewpoint, for example, when methylation of DNA is measured in medical facilities, a rapid and accurate analysis is desired. However, conventional methods described below are not sufficient from the above viewpoints.

As known methods of analyzing DNA methylation, particularly, a method of analyzing 5-methylcytosine, there may be mentioned, for example,

- (1) a method in which a DNA sample is decomposed into bases with an enzyme, and the resulting bases are analyzed by chromatography or an ELISA method,
- (2) a method in which cytosine bases contained in a DNA sample are converted to uracil with an appropriate agent such as bisulfite, and a PCR is carried out using a PCR primer specifically hybridizing with a methylated DNA or a PCR primer specifically hybridizing with a unmethylated DNA,
- (3) a method of directly sequencing a DNA sequence of the DNA previously treated with bisulfite, in a manner similar to that of the above method of (2), or
- (4) a bisulfite-PCR-SSCP (single strand conformational polymorphism) method (non-patent reference 1). In the above method (2), the presence or absence of methylation at the region to be hybridized with each primer may be judged on the basis of an appearance of each PCR product.

[0005]

These known methods can be used in analyzing methylation of a specific cytosine base, or plural cytosine bases in a specific region, but cannot be used for a comprehensive analysis of methylation of genomic DNA.

[0006]

Patent reference 1 discloses a DNA array capable of comprehensively analyzing sites to be methylated in genomic DNA. Hereinafter, the site to be methylated is referred to as a methylation site, and the site which is actually methylated is referred to as a methylated site. The term "DNA array" as used herein includes, for example, a DNA microarray, a DNA chip, or a biochip.

[0007]

Patent reference 1 discloses a DNA array in which plural kinds of DNA fragments (or chemically synthesized oligonucleotide probes having partial nucleotide sequences thereof) obtained by digesting genomic DNA with a methylation-insensitive restriction enzyme (for example, XmaI), in which a methylation-sensitive restriction enzyme (for example, SmaI) capable of digesting the same recognition site is known, are independently immobilized on

a substrate, and a method of detecting methylation in methylated sites using the same. The term "methylation-insensitive restriction enzyme" as used herein means a restriction enzyme capable of digesting a recognition site regardless of the presence or absence of methylation in the recognition site. The term "methylation-sensitive restriction enzyme" as used herein means a restriction enzyme in which the digestion activity is affected by the presence or absence of methylation in the recognition site. [0008]

The detection method disclosed in patent reference 1 comprises the steps of:

- (1) digesting genomic DNA to be analyzed with a methylation-sensitive restriction enzyme (such as SmaI, which generates a blunt end and cannot digest a recognition sequence when methylated cytosine is contained in the recognition sequence),
- (2) digesting the resulting genomic DNA of the above step (1) with a methylation-insensitive restriction enzyme capable of digesting the same recognition site but generating a different cleavage end (such as XmaI, which generates a cohesive end having a 5' overhang),
- (3) specifically amplifying only DNA fragments having cleavage ends generated by the methylation-insensitive restriction enzyme at both ends, and
- (4) detecting the amplified DNA fragments using the DNA array. As the method of specifically amplifying only the specific DNA fragments (i.e., DNA fragments having cleavage ends generated by the methylation-insensitive restriction enzyme at both ends) in the above step (3), a method in which an XmaI adaptor is ligated with the DNA fragment and a PCR method is carried out using a primer having a partial nucleotide sequence of the adaptor is disclosed.

[0009]

In the detection method disclosed in patent reference 1, since genomic DNA is digested with a methylation-sensitive restriction enzyme (the recognition cannot be digested when methylated cytosine is contained in the recognition sequence) followed by a methylation-insensitive restriction enzyme, the recognition site digested with the methylation-insensitive restriction enzyme always contains methylated cytosine. Both ends of DNA fragments amplified in the step

(3) are derived from the recognition site containing methylated cytosine, i.e., methylated methylation site (methylated site), and only DNA fragments interposed between methylated sites are detected.

[0010]

As above, in the detection method disclosed in patent reference 1, only DNA fragments in which both methylation sites at both ends are methylated can be detected, but DNA fragments in which either methylation site is methylated cannot be detected, and thus, there is a possibility that a slight change of methylation in a living body is overlooked. For example, it is frequently observed in cancer cells that CpG islands having a low methylation ratio in general are hypermethylated. Such a slightly increased methylation in a DNA fragment not methylated in general (for example, methylation in either end of a DNA fragment) cannot be detected by the detection method disclosed in patent reference 1.

[0011]

In the detection method disclosed in patent reference 1, since the two-step digestion with restriction enzymes is essential, the digestion takes time. Although it is preferable to carry out a desalting treatment after the digestion, because a ligation efficiency in the adaptor-ligation treatment is decreased when a salt concentration is high, but the procedure of desalting is complicated. In PCR commonly used in a DNA amplification, when DNA fragments to be amplified vary in size, it is known that shorter DNA fragments are generally amplified in preference to longer DNA fragments, and thus, it is difficult to amplify all DNA fragments at the same efficiency.

[0012]

In the DNA array disclosed in patent reference 1, plural kinds of DNA fragments (or chemically synthesized oligonucleotide probes) obtained by digesting genomic DNA with a methylation-insensitive restriction enzyme (such as XmaI) are independently immobilized on a substrate. However, each DNA fragment or oligonucleotide probe does not have a special feature.

[0013]

[Non-patent reference 1] M.Maekawa et al., Biochemical and Biophysical Research Communications, Netherlands, 1999, vol.

62, p. 671-676

[Patent reference 1] Japanese Unexamined Patent Publication No. 2003-38183 (JP 2003-38183 A1)

[DISCLOSURE OF INVENTION]

[PROBLEMS TO BE SOLVED BY THE INVENTION]

[0014]

An object of the present invention is to remedy the above-mentioned disadvantages of the prior art, and to provide a DNA array for analyzing a DNA methylation, which enables an exhaustive analysis of methylation sites in a DNA (for example, genomic DNA), a method of producing the DNA array, and a method of analyzing DNA methylation.

More particularly, an object of the present invention is to provide an DNA array and a method of analyzing DNA methylation, which enable a detection of not only a DNA fragment in which both methylation sites located at both ends are methylated, but also a DNA fragment in which only one of the methylation sites at both ends is methylated or a DNA fragment in which no methylation sites at both ends are methylated, without complicated procedures. Another object is to provide a method of producing a DNA array, which enables a classifying of nucleic acids to be arranged on an array into appropriate groups by taking into consideration a state of methylation sites at both ends.

[MEANS FOR SOLVING THE PROBLEMS]

[0015]

The above objects can be solved, according to the present invention, by a method of producing a DNA array, characterized by comprising the steps of:

- (1) preparing a mixture of DNA fragments in which cytosine to be methylated or methylated cytosine is exposed (hereinafter referred to as preparation step),
- (2) bringing the mixture of DNA fragments obtained in step (1) into contact with an antibody specific to cytosine or an antibody specific to methylated cytosine, and separating the mixture into a group consisting of DNA fragments which form an immunocomplex with the antibody and another group consisting of DNA fragments which do not react with the antibody (hereinafter referred to as antibody-contact step),
- (3) identifying all or part of DNA fragments contained in each of the DNA fragment groups (hereinafter referred to as identification step), and

(4) arranging one or more nucleic acids capable of hybridizing with any one of the identified DNA fragments on a substrate (hereinafter referred to as arrangement step).
[0016]

According to a preferred embodiment of the method of the present invention, the contact of the DNA fragment mixture with the antibody in the antibody-contact step is carried out under conditions in which a monovalent binding is dissociated and a divalent binding is maintained in the antigen-antibody reaction.

[0017]

According to a preferred embodiment of the method of the present invention, the mixture of DNA fragments in which cytosine to be methylated or methylated cytosine is exposed is prepared by digesting genomic DNA with "a restriction enzyme which can digest a DNA regardless of the presence or absence of methylation in a recognition site to generate a cohesive end containing cytosine to be methylated or methylated cytosine" in the preparation step.

According to another preferred embodiment of the method of the present invention, the mixture of single-stranded DNA fragments or partially single-stranded DNA fragments in which cytosine to be methylated or methylated cytosine is exposed is prepared by fragmenting genomic DNA and rendering the fragmented genomic DNAs fully or partially single-stranded in the preparation step.

According to still another preferred embodiment of the method of the present invention, a mixture of free DNA fragments contained in a biological sample or a sample derived therefrom is used as the mixture of DNA fragments.
[0018]

The present invention relates to a DNA array obtainable by the above method.

The present invention relates to a group of DNA fragments, characterized by comprising only any one of
(1) a DNA fragment having cohesive ends containing cytosine to be methylated or methylated cytosine at both ends, wherein methylated cytosine is contained in both of the cohesive ends,

(2) a DNA fragment having cohesive ends containing cytosine to be methylated or methylated cytosine at both ends, wherein methylated cytosine is contained in only one of the

cohesive ends, or

(3) a DNA fragment having cohesive ends containing cytosine to be methylated or methylated cytosine at both ends, wherein no methylated cytosine is contained in both of the cohesive ends.

The present invention relates to a DNA array characterized in that one or more nucleic acids capable of hybridizing with all or part of DNA fragments contained in the above group of DNA fragments are arranged on a substrate.

[0019]

The present invention relates to a method of analyzing DNA methylation in a DNA to be assayed, characterized by comprising the steps of:

(1) preparing a mixture of DNA fragments in which cytosine to be methylated or methylated cytosine is exposed, from the DNA to be assayed (hereinafter referred to as preparation step),

(2) bringing the mixture of DNA fragments obtained in the step (1) into contact with an antibody specific to cytosine or an antibody specific to methylated cytosine, and separating the mixture into a group consisting of DNA fragments which form an immunocomplex with the antibody and another group consisting of DNA fragments which do not react with the antibody (hereinafter referred to as antibody-contact step), and

(3) analyzing DNA fragments contained in the DNA-fragment group which forms an immunocomplex with the antibody or the DNA-fragment group which does not react with the antibody, using a DNA array (hereinafter referred to as analysis step).

[0020]

The present invention relates to a method of purifying a double-stranded DNA fragment having a cohesive end, characterized by bringing the double-stranded DNA fragment into contact with an antibody specific to a base contained in the cohesive end.

[0021]

The term "cytosine" as used herein means cytosine not methylated, i.e., unmethylated cytosine. The term "methylated cytosine" as used herein means 5-methylcytosine.

The term "antibody specific to methylated cytosine" or "anti-methylated cytosine antibody" as used herein means an antibody which specifically reacts with methylated cytosine, but does not specifically react with cytosine, unless otherwise specified. The term "antibody specific to cytosine" or "anti-cytosine antibody" as used herein means an antibody which specifically reacts with cytosine, but does not specifically react with methylated cytosine, unless otherwise specified.

[0023]

The term "antibody" as used herein includes a monoclonal antibody and a polyclonal antibody. The term "antibody" includes not only an antibody in a narrow sense (i.e., an immunoglobulin molecule per se), but also a fragment of an antibody, such as Fab, Fab', F(ab')₂, or Fv.

[EFFECTS OF THE INVENTION]

[0024]

According to the method of the present invention for analyzing methylation, on the basis of trends of positive spots and/or negative spots on the DNA array (i.e., numbers thereof) in each cell to be analyzed, the methylation ratio in genomic DNA of the cell can be evaluated. Further, on the basis of profiles of positive spots and/or negative spots, the state of the cell (for example, malignancy of a cancer cell, differentiation of a cell, or morbidity of diseases) can be evaluated.

[0025]

According to the production method of the present invention, the DNA array of the present invention, which may be used in the method of the present invention for analyzing methylation, can be produced. According to the production method of the present invention, DNA fragments obtained by digesting genomic DNA from a cell to be analyzed with an appropriate methylation-insensitive restriction enzyme can be classified into DNA fragments in which both methylation sites at both ends are methylated, DNA fragments in which either methylation site at both ends is methylated, and DNA fragments in which both methylation sites are not methylated, and a DNA array in which the classified DNA fragments are immobilized on a substrate can be produced. According to the DNA array of the present invention, a

slight change in methylation, for example, methylation at either methylation site at both ends, can be detected.

[BEST MODE FOR CARRYING OUT THE INVENTION]

[0026]

[1] DNA array and method of producing the same according to the present invention

The method of the present invention for producing the DNA array comprises the preparation step, the antibody-contact step, the identification step, and the arrangement step.

On the basis of differences in specificities of antibodies used in the antibody-contact step, the method of the present invention for producing the DNA array includes a method (hereinafter referred to as methylated cytosine-type production method) of producing a DNA array (hereinafter referred to as methylated cytosine-type array) which can be used in analyzing DNA fragments in which at least one methylated cytosine is exposed, and a method (hereinafter referred to as cytosine-type production method) of producing a DNA array (hereinafter referred to as cytosine-type array) which can be used in analyzing DNA fragments in which at least one cytosine is exposed.

[0027]

In the methylated cytosine-type production method, included in the production method of the present invention, at least an anti-methylated cytosine antibody (i.e., an antibody which specifically reacts with methylated cytosine, but does not specifically react with cytosine) is used as the antibody used in the antibody-contact step.

In the cytosine-type production method, included in the production method of the present invention, at least an anti-cytosine antibody (i.e., an antibody which specifically reacts with cytosine, but does not specifically react with methylated cytosine) is used as the antibody used in the antibody-contact step.

In this connection, the anti-methylated cytosine antibody and anti-cytosine antibody may be used as a combination thereof in the methylated cytosine-type or cytosine-type production method.

Hereinafter, the methylated cytosine-type production method and the methylated cytosine-type DNA array will be explained, and then, the cytosine-type production method and

the cytosine-type DNA array will be explained.
[0028]

(1) Methylated cytosine-type production method and
Methylated cytosine-type DNA array

In the preparation step of the methylated cytosine-type production method of the present invention, an appropriate DNA material is used to prepare a DNA fragment mixture in which one or more methylated cytosines or one or more cytosines to be methylated are exposed in each DNA fragment.

The DNA material used in the preparation step is not particularly limited, so long as it may be analyzed by the method of analyzing methylation of the present invention, that is, it is a DNA which may contain one or more methylated cytosines or one or more cytosines to be methylated. As the DNA material, there may be mentioned, for example, genomic DNA in a cell (for example, an animal cell or a plant cell), or a mixture of free DNA fragments contained in a biological sample or a sample derived therefrom (for example, blood, plasma, serum, urine, lymph, a spinal fluid, saliva, a ascites fluid, an amniotic fluid, mucus, milk, bile, gastric juices, or an artificial dialysis fluid after dialysis).

[0029]

When genomic DNA in a cell is used, it is preferable to use a cell having a high methylation ratio, because DNA fragments to be arranged on a DNA array are selected on the basis of the presence or absence of methylated cytosine in the methylated cytosine-type production method. When the cell having a high methylation ratio is used, the DNA fragment mixture obtained in the preparation step may contain a high content of DNA fragments in which at least one methylated cytosine is exposed.

As the cell having a high methylation ratio, a cell having a methylation ratio higher than that in a normal cell, for example, cancer cells, a sperm, or seeds of plants, may be used. Only one kind of cell may be used alone, or plural kinds of cells may be used as a combination thereof. In this connection, since a methylation ratio of a normal cell varies in accordance with various conditions, for example, the type of a tissue or organ, aging, external factors, a developmental stage of an individual, differentiation, or de-differentiation, it is preferable to

select an appropriate cell used in the preparation stage, in accordance with an intended purpose of a DNA array, particularly the type of cells to be assayed.

[0030]

When genomic DNA of a cell is used as a DNA material in the preparation step of the methylated cytosine-type production method, the mixture of DNA fragments in which methylated cytosine or cytosine to be methylated is exposed may be prepared from the genomic DNA by, for example, (a) digesting the genomic DNA with a restriction enzyme which can digest a DNA regardless of the presence or absence of methylation in a recognition site (i.e., methylation-insensitive) to generate a cohesive end containing methylated cytosine or cytosine to be methylated (hereinafter referred to as C-containing cohesive end), or (b) fragmenting the genomic DNA and rendering the fragmented genomic DNAs fully or partially single-stranded to prepare a mixture of single-stranded DNA fragments or partially single-stranded DNA fragments.

[0031]

When the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end is used in the preparation step, the genomic DNA can be fragmented with the restriction enzyme to generate cohesive ends and, simultaneously, methylated cytosine or cytosine to be methylated can be exposed at the cohesive ends.

Since the C (cytosine) in a 5'-CG-3' sequence is methylated in higher animals, it is preferable to use a methylation-insensitive restriction enzyme which generates a cohesive end containing the CG sequence (hereinafter referred to as CG-containing cohesive end). As such a restriction enzyme, for example, restriction enzymes listed in Table 1 may be used. In Table 1, the underlined "C" means cytosine to be methylated, and the symbol ":" means a cleavage site. When a base represented by the symbol "Y" or "N" not underlined is C, there is a possibility that the C may be methylated. For example, a restriction enzyme BsaWI cleaves between "W" and "C" of a recognition sequence "WCCGGW" to generate a 5'-cohesive end of a 5'-CCGG-3' sequence. Further, a restriction enzyme MspI cleaves between "C" and "C" of a recognition sequence "CCGG" to generate a 5'-cohesive end of a 5'-CG-3' sequence.

[0032]

Table 1

Restriction enzyme	Recognition sequence and cleavage site	Symbol
BsaWI	W: <u>CCGGW</u>	W = A or T
BsoBI	C:YCGRG	Y = C or T, R = A or G
BssSI	C: <u>TCGTG</u>	
MspI	C: <u>CGG</u>	
TaqI	T: <u>CGA</u>	
XmaI	C: <u>CCGGG</u>	

[0033]

Since the C (cytosine) in a 5'-CNG-3' sequence is methylated in plants, it is preferable to use a methylation-insensitive restriction enzyme which generates a cohesive end containing the CNG sequence (hereinafter referred to as CNG-containing cohesive end). As such a restriction enzyme, for example, restriction enzymes listed in Table 2 may be used. In Table 2, the underlined "C" means cytosine to be methylated, and the symbol ":" means a cleavage site.

[0034]

Table 2

Restriction enzyme	Recognition sequence and cleavage site	Symbol
BsaWI	W: <u>CCGGW</u>	W = A or T
MspI	C: <u>CGG</u>	
XmaI	C: <u>CCGGG</u>	

[0035]

When the mixture of single-stranded DNA fragments or partially single-stranded DNA fragments is prepared from genomic DNA in the preparation step, an order of the step of fragmenting genomic DNA and the step of rendering genomic DNA single-stranded is not particularly limited. Either step may be carried out prior to the other, or both steps may be performed simultaneously.

[0036]

As a method of fragmenting genomic DNA, there may be mentioned, for example, a method using a restriction enzyme, a method of physically cleaving genomic DNA (for example, ultrasonication, ultraviolet irradiation, radiation irradiation, electron beam irradiation), or a method of

chemically cleaving genomic DNA.
[0037]

When a DNA (including genomic DNA and fragmented DNAs) is rendered single-stranded, the full-length of the DNA, or only a partial portion thereof may be rendered single-stranded.

[0038]

As a method of rendering the full-length of a DNA single-stranded, there may be mentioned, for example, a thermal denaturation or a treatment with a denaturing agent.

[0039]

As a method of rendering a partial portion of a DNA single-stranded, there may be mentioned, for example, a method of providing a nucleic acid (for example, an oligonucleotide or a polynucleotide; hereinafter referred to as nucleic acid for single-stranded rendering) capable of hybridizing with one chain contained in a DNA to be rendered single-stranded.

The nucleic acid for single-stranded rendering may be designed to target a portion containing one or more methylated cytosines or cytosines to be methylated in a DNA to be rendered single-stranded. Only one kind of nucleic acid may be used alone, or plural kinds of nucleic acids may be used as a combination thereof. It is preferable to use the nucleic acid for single-stranded rendering, because methylated cytosine or cytosine to be methylated in a desired specific portion can be selectively exposed.

[0040]

In some DNA fragments, one or more partial portions thereof are rendered single-stranded without an artificial treatment for single-stranded treatment (such as an addition of the nucleic acid for single-stranded rendering), because of partially forming a stem and loop structure. When such DNA fragments are used as a DNA material, the DNA fragments without such an artificial treatment may be used in the subsequent antibody-contact step, as the mixture of DNA fragments in which methylated cytosine or cytosine to be methylated is exposed.

[0041]

When the mixture of free DNA fragments contained in a biological sample or a sample derived therefrom is used as a DNA material in the preparation step of the methylated

cytosine-type production method, the mixture of DNA fragments in which methylated cytosine or cytosine to be methylated is exposed may be prepared by, for example, a method using the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end (preferably, a CG-containing cohesive end or a CNG-containing cohesive end), or a method using the nucleic acid for single-stranded rendering. When the free DNA fragments are rendered fully or partially single-stranded, the DNA fragments without an artificial treatment for single-stranded treatment may be used in the subsequent antibody-contact step, as the mixture of DNA fragments in which methylated cytosine or cytosine to be methylated is exposed.

[0042]

When the mixture of free DNA fragments contained in a biological sample or a sample derived therefrom is used as a DNA material, an amount of the free DNA fragments contained in the sample is sometimes insufficient to carry out the following steps. In such a case, a sufficient amount of the DNA fragments may be obtained by performing a DNA amplification, such as a PCR. For example, after a mixture of free DNA fragments is purified from the sample and ligated with an appropriate adaptor, a DNA amplification may be carried out using a primer specific to the adaptor.

[0043]

In the antibody-contact step of the methylated cytosine-type production method, the DNA fragment mixture obtained in the preparation step is brought into contact with an anti-methylated cytosine antibody to separate a group consisting of DNA fragments which form an immunocomplex with the antibody (i.e., complex-forming DNA fragment group) from a group consisting of DNA fragments which do not react with the antibody (i.e., unreacted DNA fragment group).

[0044]

The contact of the DNA fragment mixture with the anti-methylated cytosine antibody is not particularly limited, so long as the complex-forming DNA fragment group may be separated from the unreacted DNA fragment group. The complex-forming DNA fragment group may be separated from the unreacted DNA fragment group, for example, by bringing the DNA fragment mixture into contact with the anti-methylated cytosine antibody carried on an appropriate carrier.

Alternatively, after the DNA fragment mixture is brought into contact with the anti-methylated cytosine antibody, the complex-forming DNA fragment group is purified from the whole in accordance with a conventional method to separate the complex-forming DNA fragment group from the unreacted DNA fragment group. In the former method using an appropriate carrier, the complex-forming DNA fragment group may be separated from the unreacted DNA fragment group, for example, by an affinity chromatography using the anti-methylated cytosine antibody, or an antigen-antibody reaction using the anti-methylated cytosine antibody carried on particles separable by centrifugation or magnetic particles. In the latter two-step method, the complex-forming DNA fragment group may be purified, for example, by using a column or particles (such as magnetic particles) carrying protein A or protein G thereon.

[0045]

Among the mixture of DNA fragments in which methylated cytosine or cytosine to be methylated is exposed prepared in the preparation step, DNA fragments in which methylated cytosine is exposed form an immunocomplex together with the anti-methylated cytosine antibody, and are separated as the complex-forming DNA fragment group. DNA fragments in which methylated cytosine is not exposed do not react with the anti-methylated cytosine antibody, and are separated as the unreacted DNA fragment group.

[0046]

When the DNA fragment mixture is prepared using the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end in the preparation step, the DNA fragments have C-containing cohesive ends at both ends. When the complex-forming DNA fragment group is separated from the unreacted DNA fragment group by contact with the anti-methylated cytosine antibody, the DNA fragments contained in the complex-forming DNA fragment group include a DNA fragment in which both methylation sites located at both ends are methylated (i.e., methylated cytosine exists at both C-containing cohesive ends) and a DNA fragment in which only either methylation site at both ends is methylated (i.e., methylated cytosine exists at only either of two C-containing cohesive ends).

In this case (i.e., when the DNA fragment mixture is prepared using the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end), the contact of the DNA fragment mixture with the anti-methylated cytosine antibody may be carried out under the following specific conditions to separate a complex-forming DNA fragment group consisting of only DNA fragments in which both methylation sites located at both ends are methylated (i.e., methylated cytosine exists at both C-containing cohesive ends) from an unreacted DNA fragment group.

[0048]

It is known that a divalent binding of an antibody shows a higher affinity than a monovalent binding by a factor of approximately 10^3 (M^{-1}) in an antigen-antibody reaction [for example, Ivan Roitt, Jonathan Brostoff, and David Male; Tomio Tada, trans-ed., Menekigaku Illustrated (original, 5th ed.), February 10, 2000, Nankodo, p. 110 (original title: IMMUNOLOGY, FIFTH EDITION)]. The term "divalent binding" as used herein means that one antibody molecule (or two antibody molecules immobilized on a carrier) binds to an antigen at two antigen-binding sites. The term "monovalent binding" as used herein means that one antibody molecule binds to an antigen at one antigen-binding site.

[0049]

Hereinafter, the divalent binding and the monovalent binding will be further illustrated with reference to Figures 1 to 4. Figures 1 to 4 schematically illustrate the binding between a molecule of double-stranded DNA (1) having cohesive ends at both ends and one or two molecules of anti-methylated cytosine antibody (2). The black circle (in Figures 1 to 4) and the white circle (in Figure 1) at cohesive ends of the double-stranded DNA (1) represent methylated cytosine and cytosine, respectively.

As shown in Figures 1 to 4, a molecule of antibody (2) has two antigen-binding sites. In the monovalent binding, the antibody (2) binds to the double-stranded DNA (1) as the antigen at either antigen-binding site (Figure 1). In the divalent binding, a molecule of antibody (2) binds to a molecule of antigen (1) at two antigen-binding sites (Figure 2), or each of two antibody molecules (2) immobilized on a carrier (3) binds to an antigen molecule (1) at one antigen-

binding site (Figure 3). In Figure 4, each of two antibody molecules (2) binds to an antigen molecule (1) at one antigen-binding site, and it is known that the affinity in this case is the same as that of the monovalent binding. [0050]

As described above, the affinity of the divalent binding is different from that of the monovalent binding, and thus, it is possible to dissociate the monovalent binding and maintain the divalent binding by selecting appropriate conditions in an antigen-antibody reaction system. The binding between an antigen and an antibody is based on, for example, an electrostatic bond, a hydrogen bond, or a hydrophobic bond. It is known that the electrostatic bond may be affected by, for example, a salt concentration or a pH. Similarly, it is known that the hydrogen bond and the hydrophobic bond may be affected by, for example, a concentration of urea or guanidine hydrochloride and a concentration of polyethylene glycol, respectively. [0051]

For example, with respect to the salt concentration (for example, an NaCl concentration) in an antigen-antibody reaction system, conditions in which the divalent binding is maintained but the monovalent binding is excluded (including conditions in which the divalent binding and the monovalent binding coexist and the divalent binding dominates over the monovalent binding with respect to the ratio) vary in accordance with the kind of an antibody or a salt. The salt concentration may be easily determined in accordance with an antibody used in an antigen-antibody reaction, for example, by using experimental systems in Examples described below.

Similarly, with respect to conditions other than the salt concentration (for example, a concentration of urea, guanidine hydrochloride, or polyethylene glycol, or a pH) capable of affecting the affinity, the conditions in which the divalent binding is maintained but the monovalent binding is excluded may be easily determined in accordance with an antibody used in an antigen-antibody reaction. [0052]

When a combination of an anti-methylated cytosine antibody and an anti-cytosine antibody is used in the antibody-contact step, a DNA fragment group consisting of only DNA fragments in which only either methylation site

(i.e., cytosine) at both ends is methylated (i.e., methylated cytosine exists at only either of two C-containing cohesive ends) may be obtained as the complex-forming DNA fragment group.

For example, the DNA fragment mixture obtained in the preparation step is brought into contact with the anti-methylated cytosine antibody to separate a group consisting of DNA fragments which form an immunocomplex with the anti-methylated cytosine antibody (a complex-forming DNA fragment group) from a group consisting of DNA fragments which do not react with the anti-methylated cytosine antibody (an unreacted DNA fragment group). Then, the obtained complex-forming DNA fragment group is brought into contact with the anti-cytosine antibody to separate a group consisting of DNA fragments which form an immunocomplex with the anti-cytosine antibody (another complex-forming DNA fragment group) from a group consisting of DNA fragments which do not react with the anti-cytosine antibody (another unreacted DNA fragment group). The DNA fragment group of interest may be obtained as the another complex-forming DNA fragment group. In this connection, the contact order of the anti-methylated cytosine antibody and the anti-cytosine antibody may be reversed.

Alternately, after the antibody-contact step may be carried out using the anti-methylated cytosine antibody under ordinary conditions, an elution may be carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction to obtain the DNA fragment group consisting of only DNA fragments in which only either methylation site (i.e., cytosine) at both ends is methylated.

[0053]

As described above, one or more DNA fragment groups different in the state of methylation of cytosine contained in the C-containing cohesive ends at both ends thereof may be obtained in the antibody-contact step, by determining conditions of an antigen-antibody reaction, or selecting one or more antibodies or a combination thereof.

For example, a DNA fragment group consisting of DNA fragments in which at least one methylation site (i.e., cytosine) at both ends is methylated (i.e., methylated

cytosine exists in at least one of two C-containing cohesive ends) may be obtained as the complex-forming DNA fragment group by carrying out an antigen-antibody reaction using the anti-methylated cytosine antibody under ordinary conditions.

For example, a DNA fragment group consisting of DNA fragments in which both methylation sites (i.e., cytosine) at both ends are methylated (i.e., methylated cytosine exists at both C-containing cohesive ends) may be obtained as the complex-forming DNA fragment group by carrying out an antigen-antibody reaction using the anti-methylated cytosine antibody under conditions in which the monovalent binding is dissociated and the divalent binding is maintained.

For example, a DNA fragment group consisting of DNA fragments in which either methylation site (i.e., cytosine) at both ends is methylated (i.e., methylated cytosine exists at either of two C-containing cohesive ends) may be obtained as the complex-forming DNA fragment group by carrying out an antigen-antibody reaction using a combination of the anti-methylated cytosine antibody and the anti-cytosine antibody under conditions in which the monovalent binding is dissociated and the divalent binding is maintained.

[0054]

In the identification step of the methylated cytosine-type production method, all or part of DNA fragments contained in the complex-forming DNA fragment group, obtained in the antibody-contact step, are identified. In the identification step, information of each DNA fragment is obtained so that one or more nucleic acids used in the subsequent arrangement step may be prepared. In general, it is preferable to determine the nucleotide sequence of each DNA fragment so that the nucleic acids may be designed. Each DNA fragment cloned or the DNA fragment group per se may be used to determine each nucleotide sequence in accordance with a conventional method.

[0055]

When the antibody-contact step is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, the complex-forming DNA fragment group obtained in the antibody-contact step consists of DNA fragments in which both methylation sites at both ends are methylated (i.e., methylated cytosine exists at both C-

containing cohesive ends) (Figure 2 or Figure 3). In this case, information of DNA fragments in which both methylation sites at both ends are methylated may be obtained in the identification step.

In this connection, when the antigen-antibody reaction under the specific conditions is carried out using an antibody not immobilized on a carrier, the divalent binding may be maintained only in the case shown in Figure 2, and only information of DNA fragments in which both methylation sites at both ends are methylated and the base length is approximately 40 bp (corresponding to a distance between the antigen-binding sites of an immunoglobulin molecule) may be obtained.

[0056]

When the antigen-contact step is carried out under ordinary conditions, the complex-forming DNA fragment group obtained in the antigen-contact step contains DNA fragments in which both methylation sites at both ends are methylated (i.e., methylated cytosine exists at both C-containing cohesive ends) and DNA fragments in which either methylation site at both ends is methylated (i.e., methylated cytosine exists at only either of two C-containing cohesive ends). In this case, if the complex-forming DNA fragment group per se is used in the identification step, information of each DNA fragment may be obtained, but it is impossible to determine which DNA fragment is a DNA fragment in which both methylation sites at both ends are methylated, or a DNA fragment in which either methylation site at both ends is methylated.

[0057]

In the identification step, DNA fragments in which both methylation sites at both ends are methylated may be separated from DNA fragments in which either methylation site at both ends is methylated, on the basis of the difference in affinity between the divalent binding and the monovalent binding, to obtain information of the DNA fragments in which either methylation site at both ends is methylated. For example, after the antibody-contact step is carried out under ordinary conditions, an elution is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction. The eluted DNA fragments may be

identified to obtain information of the DNA fragments in which either methylation site at both ends is methylated.
[0058]

In the arrangement step of the methylated cytosine-type production method, one or more nucleic acids (for example, oligonucleotides or polynucleotides) capable of hybridizing with any one of the DNA fragments identified in the identification step are arranged on a substrate.

The nucleic acids may be designed and prepared on the basis of information of each DNA fragment identified in the identification step, in accordance with conventional methods for producing a DNA array.

For example, it is preferable to use oligonucleotides chemically synthesized on the basis of the nucleotide sequence of each DNA fragment, because melting temperatures (T_m) of all nucleic acids arranged on a substrate can match closely. Alternately, DNA fragments per se cloned in the identified step, or partial fragments per se derived therefrom may be arranged on a substrate. DNA fragments per se contained in the complex-forming DNA fragment group obtained in the antibody-contact step, or DNAs obtained by amplifying the DNA fragments may be arranged on a substrate.
[0059]

The nucleic acids may be arranged on a substrate in accordance with conventional methods for producing a DNA array. As a method of arranging the nucleic acids on a substrate, there may be mentioned, for example, a method of directly synthesizing oligonucleotides on a substrate, or a method of spotting previously prepared nucleic acids on a substrate to immobilize the nucleic acids thereon via, for example, covalent bonds or ionic bonds.

[0060]

In a DNA array produced by the methylated cytosine-type production method of the present invention (i.e., the methylated cytosine-type DNA array of the present invention), one or more nucleic acids capable of hybridizing with DNA fragments (among the DNA fragments contained in the DNA fragment mixture prepared in the preparation step) containing methylated cytosine in a specific region (i.e., DNA fragments in which at least one cytosine in a specific region is methylated) are arranged on a substrate. For example, when the methylation-insensitive restriction enzyme

capable of generating a C-containing cohesive end is used, the specific region is the C-containing cohesive ends at both ends. When the nucleic acid for single-stranded rendering is used, the specific region is a target region of the nucleic acid for single-stranded rendering. In a DNA fragment containing a stem and loop structure, the specific region is a loop region.

[0061]

More particularly, in a DNA array produced by the methylated cytosine-type production method of preparing the DNA fragment mixture using the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end, one or more nucleic acids capable of hybridizing with DNA fragments containing methylated cytosine in at least one of C-containing cohesive ends at both ends (i.e., DNA fragments in which at least one cytosine in at least one of C-containing cohesive ends is methylated) are arranged on a substrate. In particular, when the contact of the DNA fragment mixture with the anti-methylated cytosine antibody in the antibody-contact step is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, one or more nucleic acids capable of hybridizing with DNA fragments containing methylated cytosine in both C-containing cohesive ends at both ends are arranged on a substrate. When a combination of the anti-methylated cytosine antibody and the anti-cytosine antibody is used, or when DNA fragments obtained by performing the antibody-contact step under ordinary conditions followed by the elution under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction are identified, one or more nucleic acids capable of hybridizing with DNA fragments containing methylated cytosine in either C-containing cohesive end at both ends are arranged on a substrate.

[0062]

In a DNA array produced by the methylated cytosine-type production method of preparing the DNA fragment mixture using the nucleic acid for single-stranded rendering, one or more nucleic acids capable of hybridizing with DNA fragments containing methylated cytosine in one or more single-stranded regions (i.e., DNA fragments in which at least one

cytosine in one or more single-stranded regions is methylated) are arranged on a substrate. In particular, when the contact of the DNA fragment mixture with the anti-methylated cytosine antibody in the antibody-contact step is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, one or more nucleic acids capable of hybridizing with DNA fragments containing two or more methylated cytosine in the single-stranded region(s) are arranged on a substrate. In this connection, when the nucleic acid for single-stranded rendering is used to prepare the DNA fragment mixture, it is preferable to convert both ends of the DNA fragments into blunt ends, to exclude influence of methylated cytosine or cytosine exposed at both ends.

[0063]

In the methylated cytosine-type DNA array of the present invention, one or more nucleic acids capable of hybridizing with DNA fragments containing cytosine in a specific region (i.e., DNA fragments in which at least one cytosine in a specific region is not methylated), which may be used in the cytosine-type DNA array described below, may be arranged on a substrate. In the DNA array of the present invention, when one or more nucleic acids capable of hybridizing with DNA fragments containing methylated cytosine in a specific region, and one or more nucleic acids capable of hybridizing with DNA fragments containing cytosine in a specific region are arranged on a single substrate, methylation in plural methylation sites different in an extent of methylation can be comprehensively analyzed at a time.

[0064]

(2) Cytosine-type production method and cytosine-type DNA array

In the preparation step of the cytosine-type production method of the present invention, an appropriate DNA material is used to prepare a DNA fragment mixture in which one or more methylated cytosines or one or more cytosines to be methylated are exposed in each DNA fragment.

As the DNA material used in the preparation step, the DNA material previously mentioned in the preparation step of the methylated cytosine-type production method may be used.

In this connection, when genomic DNA in a cell is used, it is preferable to use a cell having a low methylation ratio, because DNA fragments to be arranged on a DNA array are selected on the basis of the presence or absence of cytosine in the cytosine-type production method. When the cell having a low methylation ratio is used, the DNA fragment mixture obtained in the preparation step may contain a high content of DNA fragments in which at least one cytosine is exposed.

As the cell having a low methylation ratio, there may be mentioned, for example, normal cells, ES cells, or tissue stem cells. Only one kind of cell may be used alone, or plural kinds of cells may be used as a combination thereof.

[0066]

In the preparation step of the cytosine-type production method, as a method of preparing, from a DNA material, the mixture of DNA fragments in which methylated cytosine or cytosine to be methylated is exposed, the preparation methods previously mentioned in the preparation step of the methylated cytosine-type production method may be used. The descriptions with respect to the preparation methods which may be used in the preparation step of the methylated cytosine-type production method, except for the descriptions regarding the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end, may be applied to the preparation step of the cytosine-type production method.

[0067]

When the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end is used in the preparation step of the cytosine-type production method, the genomic DNA can be fragmented with the restriction enzyme to generate cohesive ends and, simultaneously, methylated cytosine or cytosine to be methylated can be exposed at the cohesive ends.

[0068]

Since the C (cytosine) in a 5'-CG-3' sequence is methylated in higher animals, it is preferable to use a methylation-insensitive restriction enzyme which generates the CG-containing cohesive end. In the cytosine-type production method, DNA fragments to be arranged on a DNA

array are selected on the basis of the presence or absence of cytosine. Therefore, if cytosine which is not methylated is contained in the C-containing cohesive end, DNA fragments cannot be selected, because all DNA fragments contain cytosine. As such a restriction enzyme, which is not preferable, there may be mentioned, for example, BsaWI, BscBI, or XmaI listed in Table 1. As a preferable enzyme which may be used in higher animals, there may be mentioned, for example, BssSI, MspI, or TaqI listed in Table 1.

[0069]

Since the C (cytosine) in a 5'-CNG-3' sequence is methylated in plants, it is preferable to use a methylation-insensitive restriction enzyme which generates the CNG-containing cohesive end. As such a restriction enzyme, for example, restriction enzymes listed in Table 2 may be used. In BsaWI or XmaI listed in Table 2, there are two underlined "C"s to be methylated, as shown in Table 2. When BsaWI or XmaI is used and both "C"s in DNA fragments are methylated, the DNA fragments cannot react with an anti-cytosine antibody.

[0070]

In the antibody-contact step of the cytosine-type production method, the DNA fragment mixture obtained in the preparation step is brought into contact with an anti-cytosine antibody, to separate a group consisting of DNA fragments which form an immunocomplex with the antibody (i.e., complex-forming DNA fragment group) from a group consisting of DNA fragments which do not react with the antibody (i.e., unreacted DNA fragment group).

[0071]

The contact of the DNA fragment mixture with the anti-cytosine antibody is not particularly limited, so long as the complex-forming DNA fragment group may be separated from the unreacted DNA fragment group. The complex-forming DNA fragment group may be separated from the unreacted DNA fragment group, for example, by bringing the DNA fragment mixture into contact with the anti-cytosine antibody carried on an appropriate carrier. Alternatively, after the DNA fragment mixture is brought into contact with the anti-cytosine antibody, the complex-forming DNA fragment group is purified from the whole to separate the complex-forming DNA fragment group from the unreacted DNA fragment group. In

the former method using an appropriate carrier, the complex-forming DNA fragment group may be separated from the unreacted DNA fragment group, for example, by an affinity chromatography using the anti-cytosine antibody, or an antigen-antibody reaction using the anti-cytosine antibody carried on particles separable by centrifugation or magnetic particles.

[0072]

Among the mixture of DNA fragments in which methylated cytosine or cytosine to be methylated is exposed prepared in the preparation step, DNA fragments in which cytosine is exposed form an immunocomplex together with the anti-cytosine antibody, and are separated as the complex-forming DNA fragment group. DNA fragments in which cytosine is not exposed do not react with the anti-cytosine antibody, and are separated as the unreacted DNA fragment group.

[0073]

When the DNA fragment mixture is prepared using the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end in the preparation step, the DNA fragments have C-containing cohesive ends at both ends. When the complex-forming DNA fragment group is separated from the unreacted DNA fragment group by contact with the anti-cytosine antibody, the DNA fragments contained in the complex-forming DNA fragment group include a DNA fragment in which both methylation sites located at both ends are not methylated (i.e., cytosine exists at both C-containing cohesive ends) and a DNA fragment in which only either methylation site at both ends is not methylated (i.e., cytosine exists at only either of two C-containing cohesive ends).

[0074]

In this case (i.e., when the DNA fragment mixture is prepared using the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end), the contact of the DNA fragment mixture with the anti-cytosine antibody may be carried out under specific conditions to separate a complex-forming DNA fragment group consisting of only DNA fragments in which both methylation sites located at both ends are not methylated (i.e., cytosine exists at both C-containing cohesive ends) from an unreacted DNA fragment group.

With respect to the specific conditions, the descriptions, previously mentioned in the antibody-contact step of the methylated cytosine-type production method, with respect to the conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction may be applied to the specific conditions used in the cytosine-type production method. [0075]

In the antibody-contact step, one or more DNA fragment groups different in the state of methylation of cytosine contained in the C-containing cohesive ends at both ends thereof may be obtained, by determining conditions of an antigen-antibody reaction, or selecting one or more antibodies or a combination thereof.

For example, a DNA fragment group consisting of DNA fragments in which at least one methylation site (i.e., cytosine) at both ends is not methylated (i.e., cytosine exists in at least one of two C-containing cohesive ends) may be obtained as the complex-forming DNA fragment group by carrying out an antigen-antibody reaction using the anti-cytosine antibody under ordinary conditions.

For example, a DNA fragment group consisting of DNA fragments in which both methylation sites (i.e., cytosine) at both ends are not methylated (i.e., cytosine exists at both C-containing cohesive ends) may be obtained as the complex-forming DNA fragment group by carrying out an antigen-antibody reaction using the anti-cytosine antibody under conditions in which the monovalent binding is dissociated and the divalent binding is maintained.

For example, a DNA fragment group consisting of DNA fragments in which either methylation site (i.e., cytosine) at both ends is not methylated (i.e., cytosine exists at either of two C-containing cohesive ends) may be obtained as the complex-forming DNA fragment group by carrying out an antigen-antibody reaction using a combination of the anti-cytosine antibody and the anti-methylated cytosine antibody under conditions in which the monovalent binding is dissociated and the divalent binding is maintained. [0076]

The identification step and the arrangement step of the cytosine-type production method may be carried out in a manner similar to that previously described in the

identification step and the arrangement step of the methylated cytosine-type production method. The descriptions regarding the identification step and the arrangement step of the methylated cytosine-type production method may be applied to the identification step and the arrangement step of the cytosine-type production method.

[0077]

In a DNA array produced by the cytosine-type production method of the present invention (i.e., the cytosine-type DNA array of the present invention), one or more nucleic acids capable of hybridizing with DNA fragments (among the DNA fragments contained in the DNA fragment mixture prepared in the preparation step) containing cytosine in a specific region (i.e., DNA fragments in which at least one cytosine in a specific region is not methylated) are arranged on a substrate. For example, when the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end is used, the specific region is the C-containing cohesive ends at both ends. When the nucleic acid for single-stranded rendering is used, the specific region is a target region of the nucleic acid for single-stranded rendering. In a DNA fragment containing a stem and loop structure, the specific region is a loop region.

[0078]

More particularly, in a DNA array produced by the cytosine-type production method of preparing the DNA fragment mixture using the methylation-insensitive restriction enzyme capable of generating a C-containing cohesive end, one or more nucleic acids capable of hybridizing with DNA fragments containing cytosine in at least one of C-containing cohesive ends at both ends (i.e., DNA fragments in which at least one cytosine in at least one of C-containing cohesive ends is not methylated) are arranged on a substrate. In particular, when the contact of the DNA fragment mixture with the anti-cytosine antibody in the antibody-contact step is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, one or more nucleic acids capable of hybridizing with DNA fragments containing cytosine in both C-containing cohesive ends at both ends are arranged on a substrate. When a combination of the anti-cytosine antibody and the anti-

methylated cytosine antibody is used, or when DNA fragments obtained by performing the antibody-contact step under ordinary conditions followed by the elution under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction are identified, one or more nucleic acids capable of hybridizing with DNA fragments containing cytosine in either C-containing cohesive end at both ends are arranged on a substrate.

[0079]

In a DNA array produced by the cytosine-type production method of preparing the DNA fragment mixture using the nucleic acid for single-stranded rendering, one or more nucleic acids capable of hybridizing with DNA fragments containing cytosine in one or more single-stranded regions (i.e., DNA fragments in which at least one cytosine in one or more single-stranded regions is not methylated) are arranged on a substrate. In particular, when the contact of the DNA fragment mixture with the anti-cytosine antibody in the antibody-contact step is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, one or more nucleic acids capable of hybridizing with DNA fragments containing two or more cytosine in the single-stranded region(s) are arranged on a substrate. In this connection, when the nucleic acid for single-stranded rendering is used to prepare the DNA fragment mixture, it is preferable to convert both ends of the DNA fragments into blunt ends, to exclude influence of methylated cytosine or cytosine exposed at both ends.

[0080]

In the cytosine-type DNA array of the present invention, one or more nucleic acids capable of hybridizing with DNA fragments containing methylated cytosine in a specific region (i.e., DNA fragments in which at least one cytosine in a specific region is methylated), which may be used in the methylated cytosine-type DNA array previously described, may be arranged on a substrate. In the DNA array of the present invention, when one or more nucleic acids capable of hybridizing with DNA fragments containing methylated cytosine in a specific region, and one or more nucleic acids capable of hybridizing with DNA fragments containing

cytosine in a specific region are arranged on a single substrate, methylation in plural methylation sites different in an extent of methylation can be comprehensively analyzed at a time.

[0081]

[2] Method of analyzing methylation according to the present invention

The method of the present invention for analyzing methylation comprises the preparation step, the antibody-contacting step, and the analysis step. In the method of the present invention for analyzing methylation, the DNA array of the present invention, or DNA arrays other than that of the present invention may be used.

On the basis of differences in specificities of antibodies used in the antibody-contact step, the method of the present invention for analyzing methylation includes a method of analyzing DNA fragments in which at least one methylated cytosine is exposed (hereinafter referred to as methylated cytosine-type analysis method), and a method of analyzing DNA fragments in which at least one cytosine is exposed (hereinafter referred to as cytosine-type analysis method).

[0082]

In the methylated cytosine-type analysis method, included in the method of the present invention for analyzing methylation, at least an anti-methylated cytosine antibody (i.e., an antibody which specifically reacts with methylated cytosine, but does not specifically react with cytosine) is used as the antibody used in the antibody-contact step. It is preferable to use the methylated cytosine-type DNA array of the present invention as the DNA array used in the analysis step.

[0083]

In the cytosine-type analysis method, included in the method of the present invention for analyzing methylation, at least an anti-cytosine antibody (i.e., an antibody which specifically reacts with cytosine, but does not specifically react with methylated cytosine) is used as the antibody used in the antibody-contact step. It is preferable to use the cytosine-type DNA array of the present invention as the DNA array used in the analysis step.

In this connection, the anti-methylated cytosine

antibody and anti-cytosine antibody may be used as a combination thereof in the methylated cytosine-type or cytosine-type analysis method.

Hereinafter, the methylated cytosine-type analysis method will be explained, and then, the cytosine-type analysis method will be explained.

[0084]

(1) Methylated cytosine-type analysis method

In the preparation step of the methylated cytosine-type analysis method of the present invention, a DNA to be analyzed is used to prepare a DNA fragment mixture in which one or more methylated cytosines or one or more cytosines to be methylated are exposed in each DNA fragment.

The DNA to be analyzed in the methylated cytosine-type analysis method of the present invention is not particularly limited, so long as it is a DNA which may contain one or more methylated cytosines or one or more cytosines to be methylated. As the DNA to be analyzed, there may be mentioned, for example, genomic DNA in a cell (for example, an animal cell or a plant cell), or a mixture of free DNA fragments contained in a biological sample or a sample derived therefrom (for example, blood, plasma, serum, urine, lymph, a spinal fluid, saliva, a ascites fluid, an amniotic fluid, mucus, milk, bile, gastric juices, or an artificial dialysis fluid after dialysis).

[0085]

As a method of preparing the DNA fragment mixture, the preparation methods previously mentioned in the preparation step of the methylated cytosine-type production method may be used. The descriptions with respect to the preparation methods which may be used in the preparation step of the methylated cytosine-type production method may be applied to the preparation step of the methylated cytosine-type analysis method.

[0086]

In the antibody-contact step of the methylated cytosine-type analysis method, the DNA fragment mixture obtained in the preparation step is brought into contact with an anti-methylated cytosine antibody, to separate a group consisting of DNA fragments which form an immunocomplex with the antibody (i.e., complex-forming DNA fragment group) from a group consisting of DNA fragments which do not react with

the antibody (i.e., unreacted DNA fragment group).

The antibody-contact step of the methylated cytosine-type analysis method may be carried out in a manner similar to that previously described in the antibody-contact step of the methylated cytosine-type production method. The descriptions regarding the antibody-contact step of the methylated cytosine-type production method may be applied to the antibody-contact step of the methylated cytosine-type analysis method.

[0087]

In the analysis step of the methylated cytosine-type analysis method of the present invention, DNA fragments contained in the complex-forming DNA fragment group and/or DNA fragments contained in the unreacted DNA fragment group obtained in the antibody-contact step are analyzed using a DNA array, which may be appropriately selected in accordance with an intended purpose of analysis.

As previously described in the antibody-contact step of the methylated cytosine-type production method or the cytosine-type production method, various DNA fragment groups, for example;

a DNA fragment group consisting of DNA fragments in which at least one methylation site (i.e., cytosine) at both ends is methylated (i.e., methylated cytosine exists in at least one of two C-containing cohesive ends);

a DNA fragment group consisting of DNA fragments in which both methylation sites (i.e., cytosine) at both ends are methylated (i.e., methylated cytosine exists at both C-containing cohesive ends);

a DNA fragment group consisting of DNA fragments in which either methylation site (i.e., cytosine) at both ends is methylated (i.e., methylated cytosine exists at either of two C-containing cohesive ends);

a DNA fragment group consisting of DNA fragments in which at least one methylation site (i.e., cytosine) at both ends is not methylated (i.e., cytosine exists in at least one of two C-containing cohesive ends);

a DNA fragment group consisting of DNA fragments in which both methylation sites (i.e., cytosine) at both ends are not methylated (i.e., cytosine exists at both C-containing cohesive ends); or

a DNA fragment group consisting of DNA fragments in which

either methylation site (i.e., cytosine) at both ends is not methylated (i.e., cytosine exists at either of two C-containing cohesive ends) may be obtained. In the present invention, one or more DNA fragment groups may be selected in accordance with an intended purpose of analysis.

[0088]

As the DNA array used in the methylated cytosine-type analysis method of the present invention, there may be mentioned, for example, the methylated cytosine-type DNA array of the present invention, the cytosine-type DNA array of the present invention, or known DNA arrays. As known DNA arrays, there may be mentioned, for example, a DNA array in which nucleic acids capable of hybridizing with DNA fragments obtained by digesting genomic DNA with a methylation-insensitive restriction enzyme (for example, XmaI) capable of generating a cohesive end (for example, the CG-containing cohesive end) containing methylated cytosine or cytosine to be methylated are arranged in a substrate.

[0089]

The DNA fragments contained in the complex-forming DNA fragment group may be analyzed, for example, while maintaining the form as the immunocomplex, or after separating the DNA fragments from the anti-methylated cytosine antibody, so long as the analysis is not affected. In general, the anti-methylated cytosine antibody is preferably used as the antibody immobilized on an appropriate carrier.

[0090]

The analysis of the DNA fragments using the DNA array may be carried out in accordance with a conventional method. For example, the DNA fragments as a sample to be assayed are previously labeled with an appropriate label (such as a fluorescent substance or a radioactive substance), and hybridized with each nucleic acid arranged on the DNA array. Each signal derived from each labeled DNA fragment bound to each nucleic acid on the DNA array is analyzed (i.e., measured or detected) to comprehensively confirm the presence or absence of each DNA fragment capable of hybridizing with each nucleic acid. Alternately, the DNA fragments contained in the complex-forming DNA fragment group and the DNA fragments contained in the unreacted DNA fragment group may be independently labeled with different

labels to subject them to the DNA array.
[0091]

When the methylated cytosine-type DNA array of the present invention is used in the methylated cytosine-type analysis method of the present invention, for example, the DNA fragment mixture may be brought into contact with the anti-methylated cytosine antibody in the antibody-contact step to separate DNA fragments in which methylated cytosine is exposed (i.e., complex-forming DNA fragment group) from DNA fragments in which methylated cytosine is not exposed (i.e., unreacted DNA fragment group), and only the DNA fragments in which methylated cytosine is exposed may be used for the analysis using the DNA array. In this case, if a methylation ratio in genomic DNA of a cell to be analyzed is high, increased positive spots (i.e., decreased negative spots) on the DNA array are observed, because the DNA mixture contains a high content of DNA fragments in which methylated cytosine is exposed. If the methylation ratio is low, decreased positive spots (i.e., increased negative spots) on the DNA array are observed, because the DNA mixture contains a low content of DNA fragments in which methylated cytosine is exposed.

As above, on the basis of trends of positive spots and/or negative spots (i.e., numbers thereof) in each cell to be analyzed, the methylation ratio in genomic DNA of the cell can be evaluated. Further, on the basis of profiles of positive spots and/or negative spots, the state of the cell (for example, malignancy of a cancer cell, differentiation of a cell, morbidity of diseases) can be evaluated.

[0092]

When a DNA array in which nucleic acids capable of hybridizing with DNA fragments obtained by digesting genomic DNA with the methylation-insensitive restriction enzyme (for example, *Xma*I) capable of generating a cohesive end containing methylated cytosine or cytosine to be methylated (for example, CG-containing cohesive end) are arranged on a substrate is used in the methylated cytosine-type analysis method of the present invention, for example, the DNA fragment mixture may be brought into contact with the anti-methylated cytosine antibody in the antibody-contact step to separate DNA fragments in which methylated cytosine is exposed (i.e., complex-forming DNA fragment group) from DNA

fragments in which methylated cytosine is not exposed (i.e., unreacted DNA fragment group). Either group may be used for the analysis using the DNA array, or both groups independently labeled with different labels may be used for the analysis using the DNA array, to evaluate the state of methylation. If an amount of DNA fragments to be subjected to a DNA array is insufficient, a sufficient amount of DNA fragments may be obtained by previously performing a DNA amplification, such as a PCR, to carry out the analysis using a DNA array.

When the antibody-contact step is carried out under ordinary conditions, and an elution is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, the complex-forming DNA fragment group consists of DNA fragments in which both methylation sites at both ends are methylated, and the eluted unreacted DNA fragment group consists of DNA fragments in which either methylation site at both ends is methylated. These DNA fragment groups (either or both) may be subjected to the DNA array to evaluate the state of methylation in detail.

[0093]

According to the analysis method of the present invention, it is possible to analyze the state of methylation of each cell, and the kind of each cell can be mapped.

For example, almost all DNA fragments obtained by digesting genomic DNA with *Xma*I are considered to be a DNA in which methylation does not vary regardless of the kind of each cell. In particular, it is considered that parasite genes (not gene regions) should be always inactivated. It is preferable to notice a DNA fragment in which a DNA fragment derived from a cancer cell is trapped by a column, but a DNA fragment derived from a normal cell is not trapped, or a DNA fragment in which a DNA fragment derived from a normal cell is trapped by a column, but a DNA fragment derived from a cancer cell is not trapped.

[0094]

(2) Cytosine-type analysis method

In the preparation step of the cytosine-type analysis method of the present invention, a DNA to be analyzed is used to prepare a DNA fragment mixture in which one or more

methylated cytosines or one or more cytosines to be methylated are exposed in each DNA fragment.

The DNA to be analyzed in the cytosine-type analysis method of the present invention is not particularly limited, so long as it is a DNA which may contain one or more methylated cytosines or one or more cytosines to be methylated. As the DNA to be analyzed, there may be mentioned, for example, genomic DNA in a cell (for example, an animal cell or a plant cell), or a mixture of free DNA fragments contained in a biological sample or a sample derived therefrom (for example, blood, plasma, serum, urine, lymph, a spinal fluid, saliva, a ascites fluid, an amniotic fluid, mucus, milk, bile, gastric juices, or an artificial dialysis fluid after dialysis).

[0095]

As a method of preparing the DNA fragment mixture, the preparation methods previously mentioned in the preparation step of the methylated cytosine-type production method may be used. The descriptions with respect to the preparation methods which may be used in the preparation step of the methylated cytosine-type production method may be applied to the preparation step of the cytosine-type analysis method.

[0096]

In the antibody-contact step of the cytosine-type analysis method, the DNA fragment mixture obtained in the preparation step is brought into contact with an anti-cytosine antibody, to separate a group consisting of DNA fragments which form an immunocomplex with the antibody (i.e., complex-forming DNA fragment group) from a group consisting of DNA fragments which do not react with the antibody (i.e., unreacted DNA fragment group).

The antibody-contact step of the cytosine-type analysis method may be carried out in a manner similar to that previously described in the antibody-contact step of the cytosine-type production method. The descriptions regarding the antibody-contact step of the cytosine-type production method may be applied to the antibody-contact step of the cytosine-type analysis method.

[0097]

In the analysis step of the cytosine-type analysis method of the present invention, DNA fragments contained in the complex-forming DNA fragment group and/or DNA fragments

contained in the unreacted DNA fragment group obtained in the antibody-contact step are analyzed using a DNA array, which may be appropriately selected in accordance with an intended purpose of analysis.

The analysis step of the cytosine-type analysis method may be carried out in a manner similar to that previously described in the analysis step of the methylated cytosine-type analysis method. The descriptions regarding the analysis step of the methylated cytosine-type analysis method may be applied to the analysis step of the cytosine-type analysis method.

[0098]

When the cytosine-type DNA array of the present invention is used in the cytosine-type analysis method of the present invention, for example, the DNA fragment mixture may be brought into contact with the anti-cytosine antibody in the antibody-contact step to separate DNA fragments in which cytosine is exposed (i.e., complex-forming DNA fragment group) from DNA fragments in which cytosine is not exposed (i.e., unreacted DNA fragment group), and only the DNA fragments in which cytosine is exposed may be used for the analysis using the DNA array. In this case, if a methylation ratio in genomic DNA of a cell to be analyzed is high, decreased positive spots (i.e., increased negative spots) on the DNA array are observed, because the DNA mixture contains a low content of DNA fragments in which cytosine is exposed. If the methylation ratio is low, increased positive spots (i.e., decreased negative spots) on the DNA array are observed, because the DNA mixture contains a high content of DNA fragments in which cytosine is exposed.

As above, on the basis of trends of positive spots and/or negative spots (i.e., numbers thereof) in each cell to be analyzed, the methylation ratio in genomic DNA of the cell can be evaluated. Further, on the basis of profiles of positive spots and/or negative spots, the state of the cell (for example, malignancy of a cancer cell, differentiation of a cell, morbidity of diseases) can be evaluated.

[0099]

When a DNA array in which nucleic acids capable of hybridizing with DNA fragments obtained by digesting genomic DNA with the methylation-insensitive restriction enzyme (for

example, XmaI) capable of generating a cohesive end containing methylated cytosine or cytosine to be methylated (for example, CG-containing cohesive end) are arranged on a substrate is used in the cytosine-type analysis method of the present invention, for example, the DNA fragment mixture may be brought into contact with the anti-cytosine antibody in the antibody-contact step to separate DNA fragments in which cytosine is exposed (i.e., complex-forming DNA fragment group) from DNA fragments in which cytosine is not exposed (i.e., unreacted DNA fragment group). Either group may be used for the analysis using the DNA array, or both groups independently labeled with different labels may be used for the analysis using the DNA array, to evaluate the state of methylation. If an amount of DNA fragments to be subjected to a DNA array is insufficient, a sufficient amount of DNA fragments may be obtained by previously performing a DNA amplification, such as a PCR, to carry out the analysis using a DNA array.

When the antibody-contact step is carried out under ordinary conditions, and an elution is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, the complex-forming DNA fragment group consists of DNA fragments in which both methylation sites at both ends are not methylated, and the eluted unreacted DNA fragment group consists of DNA fragments in which either methylation site at both ends is not methylated. These DNA fragment groups (either or both) may be subjected to the DNA array to evaluate the state of methylation in detail.

[0100]

The main features of the methylated cytosine-type analysis method (an embodiment using the methylated cytosine-type array) and the cytosine-type analysis method (an embodiment using the cytosine-type array), which are included in the method of the present invention for analyzing methylation, are shown in Table 3.

Table 3

Analysis method	Methylated cytosine-type	Cytosine-type
Antibody used	Anti-methylated cytosine antibody	Anti-cytosine antibody
DNA array used	Methylated cytosine-type	Cytosine-type
Trends of spots	High methylation	Positive spots
	Low methylation	Negative spots
		Positive spots

[0101]

[3] Method of purifying DNA of the present invention

In the method of the present invention for purifying a DNA, a double-stranded DNA fragment having cohesive ends is obtained using an antibody specific to a base contained in the cohesive ends. The method of the present invention for purifying a DNA may be used, for example, in the antibody-contact step of the production method of the present invention or the method of the present invention for analyzing methylation.

[0102]

As a known method of purifying a DNA fragment having cohesive ends (for example, DNA fragments obtained by digesting a DNA with a restriction enzyme), for example, a method of separating DNA fragments in a gel by electrophoresis and eluting the DNA fragment of interest from the gel is commonly used. However, since the electrophoresis is an essential step in this method, the DNA fragment of interest cannot be purified rapidly.

[0103]

Various antibodies specific to each base contained in a DNA, and a method of isolating a single-stranded DNA using the same are known, but a method of isolating DNA fragments obtained by digesting a DNA with a restriction enzyme using such an antibody has never been attempted. To the knowledge of the inventor, the reasons are considered to be as follows. In a double-stranded DNA, since each base is located inside of main chains, it is considered that the binding of an anti-base antibody to the base as the antigen would be inhibited. Further, since the base length of a cohesive end generated by a restriction enzyme is generally 2 to 4 bases, it is considered that the binding of an anti-

base antibody to the base located in a cohesive end is difficult due to steric hindrance or the like.

[0104]

The present inventor examined various embodiments of the antibody-contact step in the production method of the present invention and, as a result, found that a DNA fragment having a cohesive end can be isolated by using an antibody specific to a base contained in the cohesive end. The result in which the antigen-antibody reaction at such a short cohesive end can be carried out without influence of steric hindrance or the like is unexpected.

[0105]

A double-stranded DNA fragment which may be used in the method of purifying a DNA according to the present invention is not particularly limited, so long as it has at least one cohesive end. As the double-stranded DNA fragment, there may be mentioned, for example, DNA fragments obtained by digesting a DNA with a restriction enzyme capable of generating a cohesive end, DNA fragments obtained by digesting a DNA with an exonuclease, or a double-stranded DNA fragment obtained by hybridizing two kinds of single-stranded DNAs. The base length of the cohesive end is not particularly limited, but may be, for example, 10 bases or less, preferably a base length (generally 5 bases or less, preferably 4 bases or less, more preferably 3 bases or less, most preferably 2 bases or less) of a cohesive end generated by a digestion with a restriction enzyme.

[0106]

As the base contained in a cohesive end, there may be mentioned, for example, general bases contained in a naturally-occurring DNA (for example, cytosine, methylated cytosine, adenine, guanine, or thymine), bases chemically modified or chemically converted by oxidation (for example, 8-oxoguanine, generated by an oxidation at the 8-position of the purine ring in guanine), or bases crosslinked by ultraviolet rays (for example, thymidine dimer). As the antibody specific to a base contained in a cohesive end, there may be mentioned, for example, an anti-cytosine antibody, an anti-methylated cytosine antibody, an anti-adenine antibody, an anti-guanine antibody, an anti-thymine antibody, an anti-8-oxoguanine antibody, or an anti-thymidine dimer antibody. The antibody may be selected in

accordance with a nucleotide sequence of a cohesive end of a DNA fragment to be purified.

The antibody may be a monoclonal antibody or a polyclonal antibody. The term "antibody" includes not only an antibody in a narrow sense (i.e., an immunoglobulin molecule per se), but also a fragment of an antibody, such as Fab, Fab', F(ab')₂, or Fv.

[0107]

The contact of the DNA fragment to be purified with the anti-base antibody may be carried out in a manner similar to that of a general antigen-antibody reaction. When the contact is carried out under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, a DNA fragment having the same cohesive end at both ends may be separated from the other DNA fragments. For example, when a DNA is digested with a combination of two or more kinds of restriction enzymes, a mixture of DNA fragments having various cohesive ends generated by the restriction enzymes is obtained. An antigen-antibody reaction may be carried out using the DNA fragment mixture under conditions in which the monovalent binding is dissociated and the divalent binding is maintained in an antigen-antibody reaction, to separate DNA fragments having the same cohesive end at both ends from other DNA fragments (for example, DNA fragments having different cohesive ends at both ends).

[0108]

According to the method of purifying a DNA according to the present invention, only DNA fragments containing a specific base at cohesive ends can be purified, on the basis of the presence or absence of the specific base, without performing gel electrophoresis.

For example, a plasmid containing a cloned DNA fragment is treated with two kinds of restriction enzymes A and B to excise and purify the DNA fragment. The purified DNA fragment having a cohesive end generated by the restriction enzyme A and another cohesive end generated by the restriction enzyme B is further divided with the third restriction enzyme C into two subfragments. Either subfragment may be obtained on the basis of the presence or absence of a specific base contained in a nucleotide sequence generated by the restriction enzyme A or B, without

Further, the method of purifying a DNA according to the present invention is used to amplify only one or more DNA fragments (including a case of a kind of DNA fragment and a case of a mixture of plural kinds of DNA fragments) containing a specific base in a cohesive end. Hereinafter the method of the present invention for amplifying a DNA using the method of purifying a DNA according to the present invention will be illustrated.

[0109]

In the method of amplifying a DNA according to the present invention, for example, a DNA sample is digested with a restriction enzyme capable of generating a cohesive end, and only one or more DNA fragments of interest are purified using an antibody. Since the purified DNA fragment(s) have cohesive ends, the DNA fragments may be re-ligated with a DNA ligase to generate a DNA having a high molecular weight. In this case, the purified DNA fragment(s) may be a kind of DNA fragment, or a mixture of plural kinds of DNA fragments. After the resulting high molecular weight DNA is amplified using an appropriate DNA amplification system, the amplified DNA may be re-digested with the restriction enzyme used in the first digestion to selectively amplify only DNA fragment(s) previously purified by using the antibody.

[0110]

As the DNA amplification system, there may be mentioned, for example, an amplification system using GenomiPhi (for example, GenomiPhi DNA Amplification Kit, Cat. #25-6600-01; Amersham). According to the amplification system, a DNA having a base length of 50 kbp or more can be efficiently amplified. For example, several nanograms of DNA can be amplified to several micrograms of DNA. Since random primers are used, all DNA fragments previously purified by using the antibody can be uniformly amplified. The DNA amplification system is not particularly limited, so long as all DNA fragments obtained may be amplified at a time. The same result may be obtained, for example, by ligating a linker adaptor to the DNA fragments purified by using the antibody and amplifying the ligated DNA fragments by a PCR method using oligo-DNA primers specific to the linker adaptor.

According to the method of amplifying a DNA according to the present invention, a high S/N ratio can be obtained, in comparison with a DNA amplification using DNAs containing various genes as a template, because the DNA(s) of interest are concentrated before the amplification. This indicates that the content of the DNA(s) of interest in the amplified DNAs can be easily judged.

Until now, an amplified DNA product in which the state of methylation of cytosine in a template DNA is maintained has not been obtained. However, in the present invention, the DNA(s) of interest are concentrated and purified by the antibody using, as an index, methylation of cytosine in cohesive ends (i.e., single-stranded DNA regions) at both ends generated by a digestion of a restriction enzyme, before the DNA amplification, and thus, only methylated DNA(s) can be amplified.

As above, even if a large amount of DNA fragments in which cytosine in single-stranded cohesive ends is not methylated are contained in a DNA sample to be assayed, DNA fragments in which cytosine in single-stranded cohesive ends is methylated can be efficiently amplified.

[0112]

For example, in a patient suffering from a cancer, a very small amount of cancer-specifically methylated DNA fragments flow from a cancer tissue into blood. In a healthy person not suffering from a cancer, several nanograms/mL of DNA fragments derived from normal cells flow in blood. To analyze whether or not a subject is suffering from a cancer, it is necessary to detect abnormal methylated DNA fragments derived from a cancer in the DNA fragments derived from normal cells.

According to the present invention, cancer-specifically methylated DNA fragments can be concentrated using an antibody. In the concentrated and purified DNA fraction, DNA fragments having abnormal methylation caused by canceration are concentrated.

[EXAMPLES]

[0113]

The present invention now will be further illustrated by, but is by no means limited to, the following Examples.
[Example 1: Production of methylated cytosine-type DNA

(1) Separation of XmaI-digested DNA fraction

Genomic DNA was extracted from a human normal fibroblast TIG-1 [population doubling level (PDL) = 29 to 30] in accordance with a conventional method, and 0.5 μ g of genomic DNA was digested with a restriction enzyme XmaI (10 U) for 2 hours. The digested DNA fragments were diluted with 1 mL of 10 mmol/L phosphate buffer (PB)/50 mmol/L NaCl/0.05% Tween 20 (pH 7.15) to dilute a reducing agent contained in a buffer for a restriction enzyme. To the solution, 10 μ g of an anti-5-methylcytosine monoclonal antibody was added, and the whole allowed to stand at room temperature for 5 minutes. After the reaction, the whole was passed through a protein A column [CIM monolithic column Protein A HLD; BIA Separations d.o.o. (Slovenia); <http://www.monoliths.com/>]. The column was washed twice with 10 mmol/L PB/0.15 mol/L NaCl/0.05% Tween 20 (2.5 mL) to remove non-adsorbed DNA fragments from the column. Next, 10 mmol/L PB/0.4 mmol/L NaCl/0.05% Tween 20 (4 mL) was passed through the column to elute DNA fragments, which had monovalently bound to the antibody, from the column, and the fraction was kept as a monovalent-binding DNA fraction. After the column was washed with the same buffer (5 mL), all remaining DNA fragments were eluted with 2 mL of 0.3 mol/L sodium acetate (pH 4.5), and the fraction was kept as a divalent-binding DNA fraction.

[0114]

A total amount of each DNA fraction was measured, and the monovalent-binding and divalent-binding DNA fractions were 16.7 ng and 7.2 ng, respectively. In this connection, 10 μ g of antibody was used in this example. When an amount of antibody to be added is increased, an amount of DNA which binds to the column is increased and, as a result, an increased yield of DNA may be obtained. Further, when a length of the column is lengthened, an increased yield of DNA may be obtained.

[0115]

(2) Amplification and cloning of DNA fragments and production of DNA array

To each fraction obtained in Example 1(1), 1/10 volume of 3 mol/L sodium acetate and an 2.5-fold amount of ethanol are added. The mixture is allowed to stand at -20°C for an

hour, and centrifuged to collect DNAs as a precipitate. The precipitated DNAs are ligated in a conventional method to generate DNAs having a high molecular weight. The DNAs are amplified using a commercially available amplification system (GenomiPhi DNA Amplification Kit, Cat. #25-6600-01; Amersham). The amplified DNAs are redigested with XmaI, and each DNA fragment contained therein is cloned and sequenced. Oligonucleotides to be arranged on a DNA array are designed and chemically synthesized on the basis of the determined nucleotide sequences, taking Tm into consideration. The synthesized oligonucleotides are arranged on a substrate in a conventional method to produce the DNA array of the present invention.

[INDUSTRIAL APPLICABILITY]

[0116]

The DNA array of the present invention and the method of the present invention for analyzing DNA methylation may be used in analyzing DNA methylation.

[BRIEF DESCRIPTION OF THE DRAWINGS]

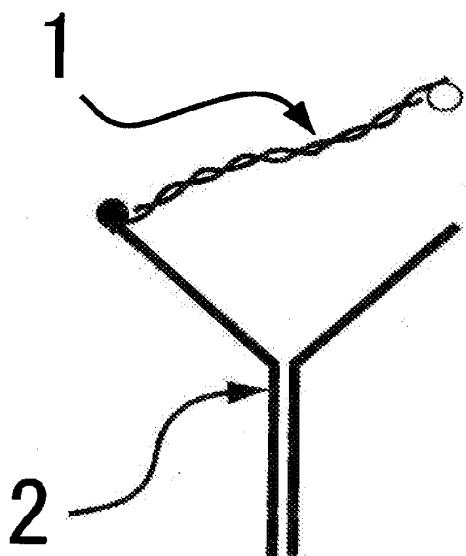
[0117]

Figure 1 schematically illustrates the state of a monovalent binding in which an antibody molecule binds to a double-stranded DNA via one antigen-binding site.

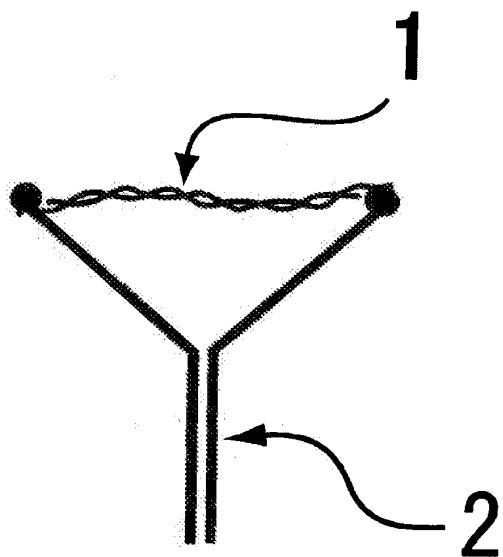
Figure 2 schematically illustrates the state of a divalent binding in which an antibody molecule binds to a double-stranded DNA via two antigen-binding sites.

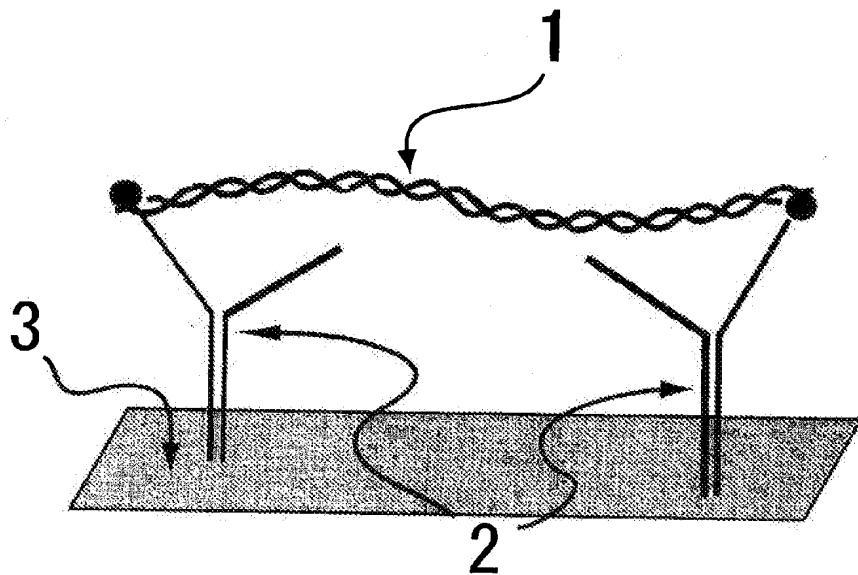
Figure 3 schematically illustrates the state of a divalent binding in which each of two antibody molecules immobilized on a carrier binds to a double-stranded DNA via one antigen-binding site.

Figure 4 schematically illustrates the state in which each of two antibody molecules binds to a double-stranded DNA via one antigen-binding site.

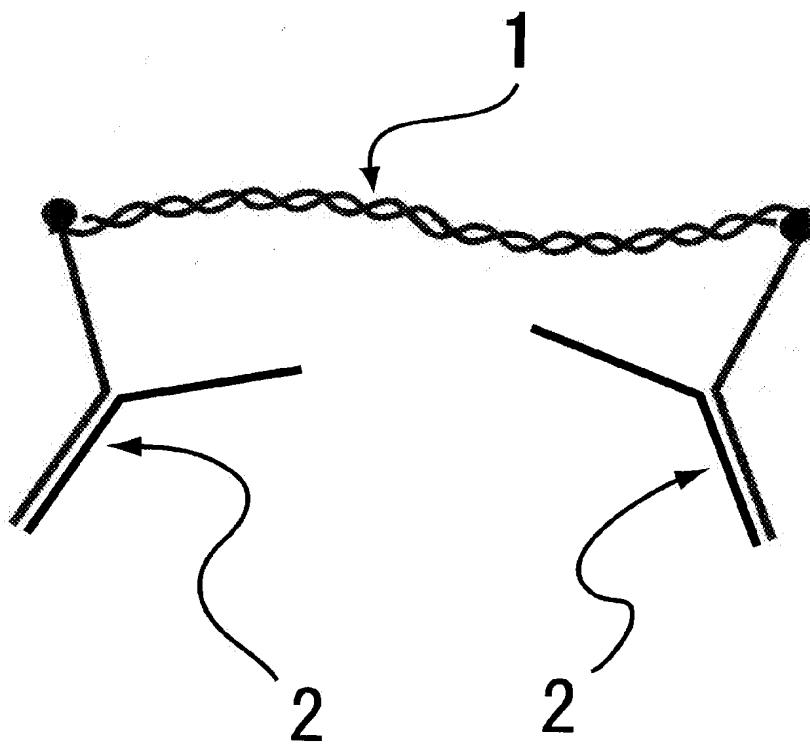


[Figure 2]





[Figure 4]



[DOCUMENT NAME] Abstract

[ABSTRACT]

[OBJECT] A DNA array for analyzing a DNA methylation, which enables an exhaustive analysis of methylation sites in a DNA (for example, genomic DNA), a process of producing the DNA array, and a method of analyzing DNA methylation are provided.

[MEANS FOR SOLUTION] The process comprises the steps of:

- (1) preparing a mixture of DNA fragments in which cytosine or methylated cytosine is exposed,
- (2) bringing the mixture of DNA fragments into contact with an antibody specific to cytosine or an antibody specific to methylated cytosine, and separating the mixture into a immunocomplex-forming DNA-fragment group and an unreacted DNA-fragment group,
- (3) identifying all or part of DNA fragments contained in the immunocomplex-forming DNA-fragment group, and
- (4) arranging one or more nucleic acids capable of hybridizing with the identified DNA fragments on a substrate. The DNA array can be obtained by the process. The analysis method can be performed by using, for example, the DNA array.

[SELECTED DRAWINGS] None